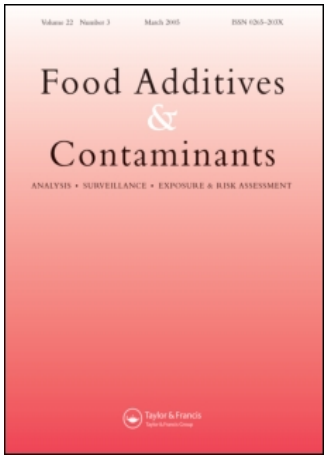

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J. R. Wilkinson [a]; J. Yu [b]; H. K. Abbas [c]; B. E. Scheffler [d]; H. S. Kim [e]; W. C. Nierman [fg]; D. Bhatnagar [b]; T. E. Cleveland [b]
[a] Department of Biochemistry and Molecular Biology, Mississippi State, MS 39762, USA
[b] USDA/ARS, Southern Regional Research Center, New Orleans, LA 70124, USA
[c] USDA/ARS/MSA, National Biological Control Laboratory, Stoneville, MS 38776, USA
[d] USDA/ARS/MSA, Jamie Whitten Delta States Research Center, Stoneville, MS 38776, USA
[e] Department of Medicine, Korea University, Sungbuk-ku, Seoul 136-705, South Korea
[f] The Institute for Genomic Research, Rockville, MD 20850, USA
[g] Department of Biochemistry and Molecular Biology, The George Washington University School of Medicine, Washington, DC 20037, USA

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Aflatoxin formation and gene expression in response to carbon source media shift in *Aspergillus parasiticus*

J. R. WILKINSON[1], J. YU[2], H. K. ABBAS[3], B. E. SCHEFFLER[4], H. S. KIM[5], W. C. NIERMAN[6,7], D. BHATNAGAR[2], & T. E. CLEVELAND[2]

[1]*Department of Biochemistry and Molecular Biology, P. O. Box 9650, Mississippi State, MS 39762, USA,* [2]*USDA/ARS, Southern Regional Research Center, 1100 Robert E. Lee Blvd, New Orleans, LA 70124, USA,* [3]*USDA/ARS/MSA, National Biological Control Laboratory, 59 Lee Road, Stoneville, MS 38776, USA,* [4]*USDA/ARS/MSA, Jamie Whitten Delta States Research Center, 141 Experiment Station Road, Stoneville, MS 38776, USA,* [5]*Department of Medicine, Korea University, 126-1, 5-ka, Anam-dong, Sungbuk-ku, Seoul 136-705, South Korea,* [6]*The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850, USA, and* [7]*Department of Biochemistry and Molecular Biology, The George Washington University School of Medicine, 2300 Eye Street NW, Washington, DC 20037, USA*

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Abstract
Aflatoxins are toxic and carcinogenic polyketide metabolites produced by fungal species, including *Aspergillus flavus* and *A. parasiticus*. The biosynthesis of aflatoxins is modulated by many environmental factors, including the availability of a carbon source. The gene expression profile of *A. parasiticus* was evaluated during a shift from a medium with low concentration of simple sugars, yeast extract (YE), to a similar medium with sucrose, yeast extract sucrose (YES). Gene expression and aflatoxins (B_1, B_2, G_1, and G_2) were quantified from fungal mycelia harvested pre- and post-shifting. When compared with YE media, YES caused temporary reduction of the aflatoxin levels detected at 3-h post-shifting and they remained low well past 12 h post-shift. Aflatoxin levels did not exceed the levels in YE until 24 h post-shift, at which time point a tenfold increase was observed over YE. Microarray analysis comparing the RNA samples from the 48-h YE culture to the YES samples identified a total of 2120 genes that were expressed across all experiments, including most of the aflatoxin biosynthesis genes. One-way analysis of variance (ANOVA) identified 56 genes that were expressed with significant variation across all time points. Three genes responsible for converting norsolorinic acid to averantin were identified among these significantly expressed genes. The potential involvement of these genes in the regulation of aflatoxin biosynthesis is discussed.

Keywords: Aspergillus, *norsolorinic acid*, *averantin*, *aflatoxins*, *carbon medium*, *microarray*

Introduction

Aflatoxins are an extremely toxic group of mycotoxins that occur as natural contaminants in agricultural oil seed products such as corn, cotton, peanut and treenuts. Aflatoxins, produced mainly by the fungi *Aspergillus flavus* and *A. parasiticus*, were identified as the most potent natural hepatocarcinogenic compounds known (Bressac et al. 1991; Hsu et al. 1991; Wogan 1992). The majority of *A. flavus* strains (or isolates) produce only aflatoxin B_1 and B_2, while the majority of *A. parasiticus* strains (or isolates) produce aflatoxins G_1 and G_2 in addition to B_1 and B_2. Aflatoxin contamination poses a serious health hazard to animals and humans, and intensive research into the chemistry, enzymology, and genetics of aflatoxin biosynthesis has been initiated (Yu et al. 2002, 2004a; Bhatnagar et al. 2003). With the release of the *A. flavus* EST sequences and the availability of the *A. flavus* amplicon microarrays (OBrian et al. 2003; Yu et al. 2004b), functional

Correspondence: J. R. Wilkinson. E-mail: jwilkinson@bch.msstate.edu

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genomic studies on the regulation of aflatoxin biosynthesis with respect to environmental parameters became possible.

The production of aflatoxin is affected by both nutritional and environmental factors (Buchanan and Stahl 1984; Luchese and Harrigan 1993; Keller et al. 1997; Feng and Leonard 1998; Payne 1998; Payne and Brown 1998; Yu et al. 2003). In maize, *Aspergilli* colonization is favoured during plant stress and the levels of aflatoxin increase with drought, heat, nitrogen deficiency, as well as with an increased pressure of insects and weeds (Darrah and Barry 1991). In an effort to identify the mechanisms responsible for inducing aflatoxin production, many abiotic and biotic factors have been investigated. Among those factors found to have an effect are carbohydrates (Abdollahi and Buchanan 1981a, b; Buchanan and Stahl 1984; Wiseman and Buchanan 1987), various nitrogen sources (Naik et al. 1970; Payne and Hagler 1983), metals (Tiwari et al. 1986; Cuero et al. 2003), lipids (Fanelli et al. 1981; Buchanan and Stahl 1984; Yu et al. 2003), and amino acids (Payne and Hagler 1983).

Secondary metabolism is also linked to developmental regulation, which is a complex event linked to several gene products and factors (Calvo et al. 2002). One of the most influential factors is an available carbon source (Davis and Diener 1968; Buchanan and Stahl 1984). Simple sugars are more favourable for aflatoxin biosynthesis. Thus, media containing 0.1 M or higher concentrations of a suitable carbohydrate source will support biosynthesis of aflatoxin (Wiseman and Buchanan 1987). However, no systematic investigation on gene expression profiling has been reported. Using microarray technology, we were able to study the patterns of gene expression in response to carbohydrate by shifting growth media from yeast extract (YE) to YE supplemented with sucrose (YES). In this study, the significantly up and down-regulated genes have been reported and potential targets for inhibiting aflatoxin formation have been identified and discussed.

Materials and methods

Culture conditions

Aspergillus parasiticus SRRC 143 ATCC 56775 (or SU-1), a wild-type strain previously used for biochemical and genetic studies of both B group (B_1 and B_2) and G group (G_1 and G_2) aflatoxins, was selected for the study. Fresh conidia were generated by plating 50 µl of 10^8 spore ml^{-1} onto Difco potato-dextrose agar (PDA) (American Scientific Products, Charlotte, NC, USA) and incubated at 30°C for 5 days. The conidia were collected from 5-day cultures with sterile aqueous 0.05% Triton X-100.

Aspergillus parasiticus spores were inoculated to a final concentration of 10^5 spores ml^{-1} in 200 ml YE liquid medium consisting of 25 g l^{-1} of yeast extract (DIFCO) and incubated for 48 h at 30°C with constant shaking at 150 rpm. The fungal mycelia were harvested by filtration *in vacuo*. The harvested mycelia were divided into 1 g aliquots. Each aliquot was used to inoculate 100 ml of YES medium (60 g l^{-1} of sucrose and 25 g l^{-1} of yeast extract) for harvesting at different time points. The cultures shifted from YE to YES were grown at 30°C with 150 rpm shaking until harvested at 3, 6, 12, 24, and 48 h post-inoculation. The harvested fungal mycelial samples were fresh frozen in liquid nitrogen and stored at −80°C for RNA preparation and aflatoxin extraction.

RNA extraction

The frozen fungal mycelia were ground to a fine powder in liquid nitrogen using mortar and pestle. Total RNA was extracted from approximately 100 mg of ground mycelium in 1 ml of TRIZOL® (Molecular Research Center, Inc., Cincinnati, OH, USA) according to manufacturer's instructions. Total RNA was quantified using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and visualized by agarose gel electrophoresis to ensure quality.

Aflatoxin analyses

To evaluate aflatoxin production, ground fungal tissue was weighed (typically 0.5–1.0 g) into glass scintillation vials and extracted as described by Abbas et al. (2004). After a methanol–water (70:30, v/v) extraction a 1 ml aliquot was centrifuged (12 000*g* for 10 min) and the supernatant was assayed for the presence of aflatoxins, using ELISA kits ('Veratox', Neogen Corp., Lansing, MI, USA). Additionally, 500 µl of the extract were mixed 1:1 with acetonitrile and cleaned with aluminium oxide before being analysed by HPLC (Sobolev and Dorner 2002). The operating conditions were as follows: column temperature, 38°C; flow rate, 0.9 ml min^{-1}; mobile phase, water/methanol/1-butanol (1400 + 720 + 25, v/v/v); Nova-Pak C18 column (150 × 3.9 mm; 4 µm; Waters); detector wavelength, 365 nm (excitation) and 440 nm (emission). Quantitation of aflatoxins was determined by the external standard method where the standard curve ranged from 0.5 to 20 ng ml^{-1} (AFB1, AFG1) and from 0.2 to 6 ng ml^{-1} (AFB2, AFG2) (Sigma-Aldrich, St Louis, MO, USA).

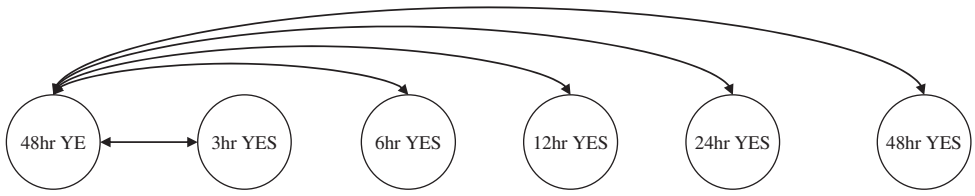

Figure 1. Microarray experimental design. Cultures of *A. flavus* were grown for 48 h in yeast extract (YE) and collected before shifting to yeast extract sucrose (YES). The 48-h YE samples were compared with 3-, 6-, 12- and 24-h YES with duplicate biological replicates and with duplicate dye-flip; the 48-h YE to 48-h YES comparisons were performed in triplicate.

Microarray design

The microarrays used were constructed at The Institute for Genomic Research (TIGR, MD, USA) with genomic DNA amplicons (approximately 530 bp) amplified using sequence-specific primers designed according to *A. flavus* EST sequence information (Yu et al. 2004b). A total of 5002 genes were arrayed at least three times each for a total of 17 991 spots. The arrays were printed by TIGR using protocols developed for human microarrays (Hegde et al. 2000) with minor modifications. PCR amplicons were purified using Millipore 96 well size exclusion vacuum filter plates. The purified PCR products were resuspended in water and diluted in a 1:1 ratio with DMSO before printing. Using an Intelligent Automation Systems spotting robot, the PCR products were spotted in triplicate at high density on Telechem Superamine aminosilane-coated microscope slides.

Hybridization

Pre-hybridization was performed according to TIGR's protocols (http://pga.tigr.org/sop/M005_1a.pdf) and washed according to post-hybridization instructions for the 3DNA Array 900MPX^{TM} Kit (Genisphere, Inc., Hatfield, PA, USA). Hybridizations were performed using the same 3DNA Array 900MPX^{TM} Kit (Genisphere), according to the manufacturer's protocol. Each experiment consisted of one 48-h YE sample coupled to either a 3-, 6-, 12-, 24-, or 48-h sample from the YE to YES shift (Figure 1). The 48-h YE to 3-, 6-, 12- and 24-h YES comparisons were repeated with duplicate biological replicates and with duplicate dye-flip; the 48-h YE to 48-h YES comparisons were done in triplicate each with a dye-flip. Briefly, 3 μg of total RNA were used to generate cDNA using Genisphere's random and MPX dT primers with Supercript II, dithiothreitol and 5X SuperScript II First Strand Buffer (Invitrogen Corp., Carlsbad, CA, USA) as per Genisphere's instructions. The generated cDNAs were purified using Qiagen MiniElute PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) as well as after terminal deoxynucleotidyl transferase tailing reaction and ligation to 3DNA capture sequence reactions. cDNA hybridizations were performed using the 2 × formamide-based hybridization buffer overnight at 49°C in hybridization cassettes (ArrayIt, TeleChem International, Inc., Sunnyvale, CA, USA), unbound cDNA was removed by washing as per Genisphere's instructions. Hybridization of the 3DNA Capture reagent containing the fluorescent dyes were performed using the 2 × formamide-based hybridization buffer at 49°C for 4 h in a hybridization cassette and washed as per Genisphere's instructions to remove non-specific background.

Hybridized slides were scanned using either a ScanArray5000XL (GSI Lumonics, Packard Biochip, Packard BioScience Co., Billerica, MA, USA) or a GenePix 4000B (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA), and the independent TIFF images from each channel were analysed using TIGR Spotfinder (http://www.tm4.org/spotfinder.html) software program. After background correction and removal of flagged values, log base 2 expression ratios were median centered and linear transformed to obtain the log and linear values, the resulting data were deposited with NCBI (GSE8038).

Data normalization and analysis

To remove the non-specific background signals, the raw data were normalized using a local regression technique LOWESS (locally weighted scatterplot smoothing), using the MIDAS software tool (http://www.tm4.org/midas.html). The LOWESS normalized dye-flips were then combined to minimize any artifacts introduced by the Cy3 or Cy5 dyes. The resulting data were averaged over duplicate gene features on each array for each replicate experiment. Some genes are represented by two separate non-overlapping fragments, therefore since a gene may be detected by each fragment they are defined as features so that gene count will not be over reported. All calculated gene expression ratios were $\log_2$-transformed when imported into TIGR MeV software (Saeed et al. 2003; http://www.tm4.org/mev.html). Only genes that gave detectable signals in all replicate experiments were used for further analysis.

Table I. Aflatoxin production at different time points after shifting *A. parasiticus* SU-1 from YE to YES medium.*

Sample	AFB_1 ($ng\,g^{-1}$)	AFB_2 ($ng\,g^{-1}$)	AFG_1 ($ng\,g^{-1}$)	AFG_2 ($ng\,g^{-1}$)	Total aflatoxin ($ng\,g^{-1}$)
48-h YE	44.9	0	5.9	0	50.8
3-h YES	2.0	0	0	0	2.0
6-h YES	6.8	0	0	0	6.8
12-h YES	8.5	0	0	0	8.5
24-h YES	440.5	11.2	490.7	0	942.4
48-h YES	475.066	14.608	511.0	0	1000.7

Notes: *Quantitation of aflatoxins was determined by high-performance liquid chromatography (HPLC) using the external standard method where the standard curve ranged from 0.5 to 20 $ng\,ml^{-1}$ (AFB_1, AFG_1) and from 0.2 to 6 $ng\,ml^{-1}$ (AFB_2, AFG_2), YE, yeast extract; YES, yeast extract sucrose.

Validation

Several environmental factors can affect the quality of the arrays: RNA extraction, labelling probes, hybridization conditions and image analysis. To ensure that genes determined to be significant by microarray analysis are reliable, it is important to show that the fold changes observed are real. To validate the expression data obtained by microarray, real-time RT-PCR (Pfaffl 2001) was performed for selected genes *aflD* (*nor-1*, an early aflatoxin biosynthetic pathway gene), *aflS* (*aflJ*, an aflatoxin regulatory gene), and TC8653 (a presumptive ubiquitin-conjugating enzyme) using the *ß-tubulin* gene as a standard (Mayer et al. 2003). The primer sequences used were: *ß-tubulin*, 5′-CGTGGT CTCAAGATGTCC-3′ and 5′-TCCATCTCGT CCATACCC-3′; *aflJ*, 5′-TCCTTACTGCAGC TAACCAATGG-3′ and 5′-CACACGGTAC TGAATCTCAAATGC-3′; *nor-1*, 5′-CACCATC ACCAACATGCAC-3′ and 5′-CTGGATCGATG ATGAAGGC-3′; and TC8653, 5′-CTAGTC AAGCTCAGTCTTCCC-3′ and 5′-CCCTTGT CATCGTTGGTC-3′. Total RNA from the 48-h YE sample and the 3-, 6-, 12-, 24-, and 48-h YES samples were DNase I (Invitrogen) treated following the manufacturer's protocols, with the addition of 0.33 µl of RNase OUT (Invitrogen) μg^{-1} of RNA. First strand cDNA synthesis was performed using the ReactionReady™ first Strand cDNA Synthesis Kit (SuperArray) with 1 µg DNase I treated total RNA; the resulting cDNAs were stored at −20°C until used for qRT-PCR. Each reaction was done in triplicate using 100 ng cDNA, 1 µM of each primer, 12.5 µl of RT^2 Real-time™ SYBR Green/Fluorescein PCR Master Mix (SuperArray Biosciences Corp., Frederick, MD, USA) and water for a 25 µl reaction volume. The PCR was performed in iCycler-iQ™ 96-well PCR Plates (BioRad Laboratories, Hercules, CA, USA) sealed with iCycler-iQ™ Optical Tape (BioRad). A 1-min run at 95°C was performed to determine dynamic well factors followed by 15 min at 95°C to activate the HotStart Taq DNA polymerase. All 50 cycles were performed according to the following temperature regimen: 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Immediately following PCR amplification, melt temperatures (T_m) of the double-stranded DNA molecules were determined by increasing the temperature from 50°C by 1°C increments every 10 s for 100 cycles.

Results

Aflatoxin production

Aspergillus parasiticus SU-1 grown in YE was found to support the biosynthesis of aflatoxins, producing 40–50 ppb total aflatoxin after 48-h growth (Table I). When mycelia collected from YE media were shifted to YES media, aflatoxin levels were initially reduced to below 20 ppb by 3 h post-shift (Table I). Total aflatoxin levels remained suppressed through 12 h post-shift. By 24 h post-shift AFB_1 and AFG_1 increased by ten- and 100-fold. The initial decrease and recovery of aflatoxin production in response to shifting from YE to YES were confirmed by both ELISA test (data not shown) and HPLC (Table I).

Transcription profile during media shift in A. parasiticus *SU-1*

Aspergillus parasiticus SU-1 grown for 48 h in YE was compared with the 3-, 6-, 12-, 24-, and 48-h post-YES shift samples by microarray experiments. A total of 2120 features were found to be expressed across all experiments as shown by Multi-Experimental Viewer analysis (TIGR MeV). These included the aflatoxin pathway genes *aflH* (*adhA*), *aflS* (*aflJ*), *aflG* (*avnA*), *aflV* (*cypX*), *aflJ* (*estA*), *aflW* (*moxY*), *aflD* (*nor-1*), *aflE* (*norA*), *aflF* (*norB*), *aflO* (*omtB*), *aflQ* (*ordA*), *aflC* (*pksA*), *aflK* (*vbs*), *aflL* (*verB*), the sugar cluster genes *glcA*, *hxtA* and *nadA*, and the putative transport gene *aflT* (Yu et al. 1995, 2004b; Chang et al. 2004). Several biosynthetic genes were detected in only a portion of the experiments, including *aflI* (*avfA*), *aflU* (*cypA*), *aflB* (*fas-1*), *hypB*, *aflP* (*omtA*), *aflX* (*ordB*),

aflN (*verA*) and the sugar utilization gene *sugR*. The regulatory gene *aflR* was not detected in any of the time point experiments. However, since both the aflatoxin pathway genes and aflatoxin production were detected, it can be presumed that *aflR* was expressed, but below the detectable level by microarray. Of the 2120 features expressed across all experiments, 56 features were found to be significant across all experiments by a one-way ANOVA test using TIGR MeV (a significance level of 0.01). The features determined to be significant can be classified into seven functional groups, aflatoxin biosynthesis, regulators, environmental information processing, genetic information processing, metabolism, secondary metabolism/degradation xenobiotics, and those of unknown function. The 53 genes represented by the 56 features are shown in functional groups in Table II, including three genes of unknown function (TC8354, TC8375, and TC8389) which are represented by two ESTs each. These duplicate ESTs show close agreement with each other and serve as additional internal controls.

qRT-PCR

Quantitative PCR was performed for cultures grown at 48 h at 30°C in YE and at 3, 6, 12, 24, and 48 h after shifting to YES. Two genes determined to be significant by microarray analysis, the aflatoxin biosynthetic gene *aflD* (*nor-1*) and TC8653, an ubiquitin-conjugating enzyme, were chosen for validation. Both, *aflD* (*nor-1*) and TC8653 expression levels are reduced after shifting to YES and remain reduced until 24 h in YES, at which point they return to levels similar to that found in the 48-h YE cultures (data not shown). Comparisons of the expression levels determined by qRT-PCR and by microarray are found to have a high correlation, 0.94 and 0.90 for TC8653 and nor-1, respectively. The regulatory gene *aflS* (*aflJ*) was also examined by qRT-PCR as a representation of genes determined to be non-significant in the array comparisons. As with *aflD* (*nor-1*) and TC8653 early repression followed by recovery of expression at the 24-h time point is observed (data not shown).

Discussion

Using a medium containing a low level of simple carbohydrate and shifting to a medium with readily available carbohydrate is commonly employed to study the induction of aflatoxin production in *Aspergilli*. This sucrose up-shift experiment appears relatively simplistic; however, in terms of genome-wide changes in gene expression it is a rather complex phenomenon. Previous authors have detected aflatoxin production as early as 16 h post-shift to a conducive medium (OBrian et al. 2003). Aflatoxin production is found to occur in both media containing a low concentration of available carbohydrate (YE) and one containing a higher amount of simple sugar (YES). However, when *Aspergillus* mycelia were shifted from a low concentration of available carbohydrate (YE) to one containing a higher amount of simple sugar (YES), aflatoxin biosynthesis is temporarily repressed. Reduction in aflatoxin levels is accompanied by a decrease in *aflD*, *aflE*, and *aflF* (*nor-1*, *norA*, and *norB*) transcripts (Table II). qRT-PCR by Scherm et al. (2005) showed that expression of *aflD* (*nor1*), *aflO* (*omtB*), and *aflP* (*omtA*) is consistently correlated with the production of aflatoxins, whereas *aflS* (*aflJ*) and *aflR* were not. As validated by qRT-PCR, *aflD* (*nor-1*) does indeed reflect changes in aflatoxin production as seen on the microarray. The validity of genes determined to be significant by ANOVA is confirmed by both the correlation seen between the microarray and qRT-PCR data for *aflD* (*nor-1*) and TC8653, and the high agreement seen in the duplicate ESTs for TC8354, TC8375, and TC8389.

Many aflatoxin biosynthetic genes were detected by the microarray experiment, though only a few were determined to be significantly different. The three aflatoxin pathway genes responsible for the conversion of norsolorinic acid to averantin (Brakhage 1998; Yu et al. 2004a), *aflD*, *aflE* and *aflF* (*nor-1*, *norA*, and *norB*) had expression levels significantly up-regulated across all experiments. However, it appears that the expression of the three genes was not influenced to the same extent by the shift, with the repression of *aflD* > *aflF* > *aflE* (*nor-1* > *norB* > *norA*). Recoveries of the expressional repression for the *aflD*, *aflE*, and *aflF* (*nor-1*, *norA*, and *norB*) to the level of the 48-h YE time point were at 12, 24 and 48 h, respectively (Table II). With the occurrence of all three genes involved in conversion of norsolorinic acid to averantin it is possible that the regulation of the committed step in the aflatoxin biosynthetic pathway is the key to aflatoxin regulation.

Of the few putative regulators of aflatoxin biosynthesis, *laeA* (Bok and Keller 2003), *veA* (Calvo et al. 2002), *aflR* (Payne et al. 1993; Woloshuk et al. 1994; Chang et al. 1995) and *aflS* (*aflJ*) (Meyers et al. 1998; Chang 2003), only the poorly understood *aflS* (*aflJ*) has been shown to modulate transcription of these pathway genes. This modulation occurs in concert with *aflR* in an as yet undefined mechanism (Chang 2003). In one recent array study, the levels of *aflR* and *aflR* antisense were relatively constant in high and low temperature cultures (Price et al. 2006), though the levels of aflatoxin production was found to vary.

Table II. Genes represented on a microarray showing significant changes in expression across time.*

Gene and/or clone ID**	Putative function	$\log_2$ 3 h[a]	$\log_2$ 6 h[a]	$\log_2$ 12 h[a]	$\log_2$ 24 h[a]	$\log_2$ 48 h[b]	p[c]
Aflatoxin biosynthesis							
aflD (nor-1) or NAGAV36TV	nor-1	−0.05 ± 0.03	−0.23 ± 0.04	0.02 ± 0.10	−0.01 ± 0.13	0.32 ± 0.09	5.11E−03
aflE (norA)	norA	0.08 ± 0.23	−0.30 ± 0.04	−0.10 ± 0.08	0.10 ± 0.12	0.48 ± 0.18	9.20E−03
aflF (norB)	norB	0.13 ± 0.06	−0.41 ± 0.13	0.16 ± 0.09	0.12 ± 0.05	0.24 ± 0.08	1.34E−03
Regulatory							
TC10887 or NAFAQ45TV	Carbon catabolite repression, CreD	1.73 ± 0.28	1.04 ± 0.22	1.63 ± 0.51	1.43 ± 0.26	−0.13 ± 0.32	5.61E−03
TC11201 or NAGAV80TV	LysR:LysR, substrate-binding	−0.12 ± 0.12	−0.31 ± 0.09	0.20 ± 0.13	−0.18 ± 0.09	0.13 ± 0.08	9.50E−03
Environmental information-processing genes							
TC10087 or NAFAX17TV	Manganese superoxide dismutase	−1.81 ± 0.23	−1.10 ± 0.13	0.27 ± 0.49	0.05 ± 0.27	0.63 ± 0.33	1.22E−03
NAFBH01TVB	Carnitine/acylcarnitine translocase	−0.09 ± 0.15	−0.52 ± 0.06	0.08 ± 0.26	0.39 ± 0.00	−0.41 ± 0.03	4.20E−03
Genetic information processing							
TC8653 or NAFAW76TV	Ubiquitin-conjugating enzyme UbcB	−0.38 ± 0.34	−0.46 ± 0.62	0.18 ± 0.29	−0.08 ± 0.13	1.46 ± 0.29	4.27E−03
TC8856 or NAGCZ42TV	Endothelin-converting enzyme, putative	−0.15 ± 0.02	−0.39 ± 0.01	0.22 ± 0.14	0.19 ± 0.12	0.24 ± 0.14	4.13E−03
TC8982 or NAFEP49TV	Ribosomal protein L30	1.09 ± 0.07	0.95 ± 0.01	0.42 ± 0.07	0.46 ± 0.46	−0.21 ± 0.12	1.81E−03
TC9250 or NAFCM63TV	Ribosomal protein P0	1.86 ± 0.51	1.26 ± 0.00	0.72 ± 0.48	0.47 ± 0.18	−0.20 ± 0.15	4.87E−03
TC10342 or NAGDF16TV	Thioredoxin a	0.10 ± 0.18	−0.37 ± 0.00	0.06 ± 0.12	−0.35 ± 0.01	−0.07 ± 0.08	9.69E−03
TC10458 or NAGAU76TV	Biosynthetic protein gpi1	−0.23 ± 0.09	−0.46 ± 0.19	0.21 ± 0.07	−0.29 ± 0.18	0.08 ± 0.08	4.41E−03
TC11185 or NAFFK71TV	Translation initiation factor 3	1.03 ± 0.17	0.56 ± 0.29	−0.38 ± 0.35	0.30 ± 0.10	−0.12 ± 0.04	3.84E−03
TC11849 or NAGCQ85TV	Histone acetyltransferase	−0.24 ± 0.14	−0.40 ± 0.09	0.10 ± 0.11	0.10 ± 0.15	0.11 ± 0.07	7.49E−03
NAGCY40TV	DNA helicase ino80	−0.26 ± 0.10	−0.49 ± 0.13	0.22 ± 0.15	0.10 ± 0.01	0.08 ± 0.05	1.97E−03
Metabolism genes							
TC8507 or NAFDE33TV	Chitosanase precursor	0.30 ± 0.03	0.75 ± 0.08	0.47 ± 0.14	−0.24 ± 0.10	−0.26 ± 0.11	1.15E−04
TC9118 or NAFBG33TVB	c-4 methyl sterol oxidase	0.63 ± 0.16	1.20 ± 0.01	0.72 ± 0.28	0.17 ± 0.42	−0.24 ± 0.17	5.08E−03
TC10159 or NAGDH86TV	NADH-ubiquinone oxidoreductase	0.22 ± 0.04	0.23 ± 0.04	0.24 ± 0.09	0.13 ± 0.10	−0.17 ± 0.06	2.72E−03
TC10311 or NAFFJ71TV	Carbonic anhydrase	−0.27 ± 0.11	−0.31 ± 0.20	0.13 ± 0.03	−0.35 ± 0.32	0.36 ± 0.10	9.91E−03
TC10654 or NAGCS41TV	Methyltransferase, putative	−0.06 ± 0.04	−0.24 ± 0.09	0.15 ± 0.14	0.36 ± 0.22	0.38 ± 0.06	7.27E−03
TC11424 or NAGCR11TV	Endoglucanase	0.22 ± 0.44	−0.07 ± 0.17	−0.27 ± 0.48	0.04 ± 0.00	2.10 ± 0.38	1.43E−03
Secondary metabolism/degradation xenobiotics genes							
TC10686 or NAFAK38TV	Oxidoreductase, Gfo/Idh/MocA family	−0.21 ± 0.04	−0.11 ± 0.14	0.18 ± 0.17	−0.03 ± 0.24	0.90 ± 0.08	6.19E−04
TC10972 or NAFCF78TV	Acid phosphatase, putative	0.07 ± 0.03	−0.10 ± 0.10	−0.09 ± 0.28	0.23 ± 0.08	1.95 ± 0.33	1.49E−04
TC10202 or NAGCR77TV	Cytochrome p450	−0.71 ± 0.04	−0.21 ± 0.30	−0.14 ± 0.14	−0.30 ± 0.36	1.30 ± 0.29	5.71E−04

TC11005 or NAFFD20TV	Myo-inositol-1-phosphate synthase	0.57 ± 0.10	0.39 ± 0.20	−0.30 ± 0.25	0.51 ± 0.13	−0.38 ± 0.26	7.71E−03
NAGDH19TV	2/3-Oxoacyl-CoA thiolase	−0.17 ± 0.03	−0.43 ± 0.03	0.04 ± 0.27	−0.11 ± 0.25	0.96 ± 0.15	1.22E−03
Genes of unknown function							
TC8353 or NAFBJ71TVB	Hypothetical protein	0.03 ± 0.41	0.42 ± 0.07	0.93 ± 0.17	0.09 ± 0.19	−0.76 ± 0.30	1.89E−03
TC8354 or NAFES85TV	Hypothetical protein	0.35 ± 0.45	0.92 ± 0.46	0.91 ± 0.25	0.00 ± 0.35	−0.83 ± 0.37	7.66E−03
TC8354 or NAFFE10TV	Hypothetical protein	0.43 ± 0.01	0.54 ± 0.10	1.09 ± 0.44	−0.04 ± 0.46	−0.85 ± 0.34	4.96E−03
TC8356 or NAFCE39TV	Hypothetical protein	0.34 ± 0.01	0.46 ± 0.02	0.92 ± 0.06	−0.18 ± 0.68	−0.91 ± 0.19	1.76E−03
TC8370 or NAFDM52TV	Hypothetical protein	−2.75 ± 0.52	−2.60 ± 0.26	−2.38 ± 0.38	−1.69 ± 0.68	0.19 ± 0.20	7.31E−04
TC8375 or NAFAB80TV	Hypothetical protein	−1.22 ± 0.42	−1.68 ± 0.28	−0.04 ± 0.69	−0.58 ± 0.23	0.99 ± 0.08	3.09E−03
TC8375 or NAGAQ37TV	Hypothetical protein	−1.56 ± 0.28	−1.38 ± 0.00	−0.04 ± 0.64	−0.53 ± 0.35	0.86 ± 0.50	7.24E−03
TC8389 or NAGDP58TV	Hypothetical protein	−3.61 ± 0.26	−2.41 ± 1.58	−0.22 ± 0.28	0.00 ± 0.00	−0.06 ± 0.41	4.06E−03
TC8389 or NAGDS58TV	Hypothetical protein	−3.52 ± 0.47	−2.46 ± 1.52	−0.04 ± 0.25	0.03 ± 0.22	−0.10 ± 0.16	2.80E−03
TC8417 or NAFFK30TV	Hypothetical protein	−0.18 ± 0.08	−0.15 ± 0.18	−0.31 ± 0.15	−0.84 ± 0.06	−0.93 ± 0.22	5.21E−03
TC8679 or NAFDD68TV	Hypothetical protein	0.95 ± 0.13	0.93 ± 0.15	0.69 ± 0.14	0.09 ± 0.48	−0.26 ± 0.24	6.03E−03
TC8793 or NAFDM75TV	Hypothetical protein	−0.19 ± 0.06	−0.27 ± 0.03	0.09 ± 0.13	0.11 ± 0.01	0.00 ± 0.05	8.84E−03
TC9112 or NAGDL82TV	Hypothetical protein	−0.85 ± 0.22	−0.61 ± 0.05	−0.08 ± 0.21	−0.31 ± 0.24	0.13 ± 0.08	5.25E−03
TC9713 or NAGDB86TV	Hypothetical protein	−0.08 ± 0.19	−0.72 ± 0.45	0.12 ± 0.10	0.14 ± 0.03	3.03 ± 0.73	3.11E−04
TC9790 or NAFDF69TV	Hypothetical protein	0.01 ± 0.06	−0.12 ± 0.03	−0.06 ± 0.25	0.49 ± 0.46	2.32 ± 0.57	1.04E−03
TC9952 or NAGDD30TV	Hypothetical protein	−0.22 ± 0.03	−0.31 ± 0.06	0.21 ± 0.22	0.01 ± 0.09	0.32 ± 0.07	9.12E−03
TC9991 or NAFFG56TV	Hypothetical protein	−0.05 ± 0.02	−0.10 ± 0.04	−0.13 ± 0.16	−0.96 ± 0.24	−1.31 ± 0.02	4.33E−05
TC10636 or NAGCO70TV	Hypothetical protein	−0.14 ± 0.02	−0.10 ± 0.02	0.29 ± 0.12	0.11 ± 0.01	−0.09 ± 0.11	7.14E−03
TC10658 or NAGCY65TV	Hypothetical protein	−0.26 ± 0.09	−0.37 ± 0.01	0.16 ± 0.05	−0.31 ± 0.03	−0.13 ± 0.04	6.92E−05
TC10865 or NAFBD80TV	Hypothetical protein	−0.14 ± 0.02	−0.29 ± 0.20	0.10 ± 0.05	−0.08 ± 0.19	−0.53 ± 0.08	6.63E−03
TC11057 or NAFEK60TV	Hypothetical protein	−0.97 ± 0.38	−0.59 ± 0.59	−0.80 ± 0.51	−0.35 ± 0.39	1.05 ± 0.07	7.91E−03
TC11210 or NAFEQ42TV	Hypothetical protein	−0.92 ± 0.23	−0.39 ± 0.08	−0.18 ± 0.17	−0.02 ± 0.05	0.05 ± 0.19	6.10E−03
TC11819 or NAFDY45TV	Hypothetical protein	−2.88 ± 0.80	−2.83 ± 0.55	−2.01 ± 0.47	−1.80 ± 0.74	0.14 ± 0.21	4.06E−03
TC12029 or NAFAX23TV	Hypothetical protein	−0.06 ± 0.08	−0.58 ± 0.18	−0.02 ± 0.12	−0.07 ± 0.01	0.03 ± 0.11	9.51E−03
NAFCJ18TV	Hypothetical protein	−0.08 ± 0.17	0.51 ± 0.12	0.97 ± 0.22	0.13 ± 0.50	−0.52 ± 0.22	4.62E−03
NAFFC54TV	Hypothetical protein	−2.61 ± 0.12	−1.50 ± 0.55	−0.47 ± 0.56	−0.04 ± 0.23	0.19 ± 0.28	1.67E−03
NAGCO36TV	Hypothetical protein	−0.72 ± 0.04	−0.76 ± 0.18	−0.13 ± 0.15	−0.99 ± 0.28	0.15 ± 0.09	8.34E−04
NAGCV91TV	Hypothetical protein	−0.57 ± 0.11	−0.44 ± 0.14	−0.18 ± 0.29	−0.16 ± 0.17	0.99 ± 0.47	8.46E−03
NAGDA95TV	Hypothetical protein	0.27 ± 0.10	0.36 ± 0.21	1.13 ± 0.32	−0.03 ± 0.59	−0.87 ± 0.46	8.83E−03

Notes: *Significance was determined by one-way analysis of variance (ANOVA) ($p = 0.01$). The values are $\log_2$ changes of expression level relative to 48-h YE media before shifting to YES.
**For sequence information of gene and EST clones, see The Institute for Genomic Research website at: http://www.tigr.org/tdb/tgi/cw/cwgi/
[a]Values are average of two replications. [b]Values are average of three replications. [c]*P*-value for genes showing significantly different expression profiles across time.

Regulation of the committed step in aflatoxin biosynthesis would explain the ambiguous results observed in most experiments with regards to *aflR*.

Significance of these norsolorinic acid genes was also seen in a recent study of the effects of tryptophan on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* (Wilkinson et al. 2007). As in this study many aflatoxin biosynthetic genes were detected in the microarray experiment, but only *aflE* (*norA*), *aflD* (*nor-1*), and *aflO* (*omtB*) were found to be significant. It is apparent that the regulation of *aflD* (*nor-1*) and *aflE* (*norA*) is of importance in both experiments. In addition to the regulation of *nor-1* expression that is thought to occur through AflR and AflJ, it has been proposed that an additional binding protein (NorLbp) exists. This DNA-specific protein complex is thought to bind onto a specific site in the *aflD* (*nor-1*) promoter termed norL (Miller et al. 2005). Changes in expression of one or more genes linked to NorLbp could directly affect aflatoxin biosynthesis and though several possible candidates exist (Table II) it may be possible to narrow down these candidates by comparing to the Trp array experiment (Wilkinson et al. 2007). In addition to *aflD* (*nor-1*) and *aflE* (*norA*), TC8370 (unknown function), TC10686 (oxidoreductase), TC11005 (myo-inositol-1-phosphate synthase), and TC10342 (thioredoxin) all show significant changes in expression in both experiments (Table II).

The NAD-dependent oxidoreductase of the Gfo/Idh/MocA family (TC10686) is thought to function in reducing fructose to sorbitol, which may serve to protect against osmotic shock (Kingston et al. 1996). Myo-inositol-1-phosphate synthase (TC11005) can catalyse the conversion of D-glucose 6-phosphate to 1L-myo-inositol-1-phosphate and is the committed step in the production of all inositol-containing compounds. Inositol is known to function in many different roles including signalling (Boss 1989; Berridege 1993) and response to osmotic stress (Sacher and Staples 1985). Thioredoxin (TC10342) is a redox-active protein that can act as an antioxidant and can be induced in response to oxidative stress (Ross et al. 2000).

The strong association of these genes to osmotic stress suggests that TC8370 may also function in osmotic stress. Osmotic stress is thought to be a prerequisite for aflatoxin production (Jayashree and Subramanyam 2000) and thus, it is likely that aflatoxin biosynthesis is controlled through these redox-related genes. The addition of anti-oxidants has been shown to reduce aflatoxin biosynthesis (Reverberi et al. 2005; Kim et al. 2006). Similarly, shifting mycelium to a fresh media with an excess carbon source appears to reduce temporarily oxidative stress that leads to aflatoxin biosynthesis.

Photosynthetic redox signals are known to be involved in regulating the Calvin cycle, ATP synthesis, and NADPH export via the ferredoxin–thioredoxin system to target enzymes that are activated by reduction of regulatory disulphide bonds (Geigenberger et al. 2005). Thioredoxins are also known to be essential for the transcriptional induction of other components encoding antioxidant proteins in *Saccharomyces cerevisiae* (Ross et al. 2000). It is quite possible that the further investigations into these uncharacterized genes identified in this report may help to unravel the regulatory mechanism of aflatoxin biosynthesis.

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